ELSEVIER

Scientia Horticulturae 80 (1999) 49–56

SCIENTIA HORTICULTURÆ

Low temperature induction of acid invertase activity in flavedo tissue of late season grapefruit (*Citrus paradisi*)[1]

James Hyde[a], George Jurch[a], Elizabeth Baldwin[b], Ed Echeverria[c,*]

[a] *Department of Chemistry, The University of South Florida, Tampa, FL 33620, USA*
[b] *USDA/ARS, Citrus and Subtropical Production Lab Research, P.O. Box 1909, Winter Haven, FL 33883, USA*
[c] *Citrus Research and Education Center, University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850, USA*

Accepted 8 September 1998

Abstract

Peel tissue from deacclimated late season grapefruit was examined for the capacity to develop cold induced acid invertase. Experimental fruit were subjected to three prolonged temperature treatments (17 days at 25°C, or 17 days at 4°C ± an additional 24 h at 25°C) and the flavedo examined for acid invertase activity immediately after the respective temperature treatment. Invertase activity was not detected when soluble extracts were assayed directly after desalting. However, after isoelectric separation, invertase activity was detected only in samples from cold treated fruit. Cell-wall pellets were also devoid of invertase activity when measured directly. After washing with high ionic buffer (containing 1 M NaCl), activity was recovered in the same cold treated samples. The data indicate that the peel of late season grapefruit retain the capacity to develop acid invertase in response to cold treatment.

Keywords: Chilling injury; Cold treatment

* Corresponding author. Tel.: +1-941-956-1151; fax: +1-941-956-4631; e-mail: eje@icon.lal.ufl.edu

[1] Florida Agricultural Experiment Station Journal Series No. R-06506.

0304-4238/99/$ – see front matter
PII: S0304-4238(98)00223-4

1. Introduction

Accumulation of reducing sugars in the peel cells of Florida grapefruit has been determined to form part of a chilling injury (CI) resistance mechanism (Purvis et al., 1979; Purvis and Grierson, 1982) that includes increases in proline concentration (Purvis and Yelenosky, 1982) and epidermal squalene (Nordby and McDonald, 1990). Sugars can function as cryoprotectants of proteins and membranes (Sakai and Yoshida, 1968; Steponkus, 1971), although high levels alone may not be sufficient to induce cold hardiness (Steponkus, 1971; Fuchigami et al., 1973). The natural CI resistance mechanism observed in grapefruit is triggered during the winter months when the mean low temperature in the field drops below 10°C (Purvis et al., 1979). The increase in reducing sugars observed in the flavedo cells is paralleled by a corresponding decline in sucrose content (Purvis and Grierson, 1982). In the spring, however, a reversal in the capacity to resist CI takes place as reducing sugars drop with a concomitant increase in sucrose content (Purvis et al., 1979). Subsequent studies by Purvis and Rice (1983) demonstrated that the fluctuations in the sucrose/reducing sugar content in the flavedo cells resulted from changes in the activity of a soluble acid invertase which develops during the winter but disappears in the spring.

Nordby and McDonald (1990) demonstrated that during late season artificial conditioning of grapefruit, the squalene content of the epidermis increased in a manner similar to in vivo during the fall months and offered a similar degree of CI protection. Whether invertase activity was also part of such artificially induced CI protection, or whether its development occurs only at a determined developmental stage remains unclear. Since 'in-transit' cold treatment of late Florida grapefruit (March–June) is recommended at low temperatures (0.6–2.2°C, Ismail et al., 1986; or 2–5°C, Wardowski, 1989; Wardowski and Brown, 1993), development of invertase activity could be crucial for CI resistance. This investigation was aimed at determining whether acid invertase activity can be induced by low temperatures in the aging peel of deacclimated late harvested grapefruit.

2. Materials and methods

White 'Marsh' grapefruit (*Citrus paradisi* Macf. 'Marsh') were randomly collected from the groves of the Citrus Research and Education Center in Lake Alfred in early August 1994. Fruit were divided into four groups (treatments), each consisting of six sets of three fruits. Treatments are described in Table 1. Each set of three fruits constituted a replicate (total of six replicates/treatment). Fruit was stored at 95% RH at the listed temperatures.

Table 1
Description of treatments to late Florida grapefruit prior to acid invertase determinations

Treatment	Description
I	Fruit assayed immediately after harvest
II	Fruit stored at 25°C for 17 days
III	Fruit stored at 4°C for 17 days
IV	Fruit stored at 4°C for 17 days and an additional 24 h at 25°C

For invertase extraction, a composite flavedo sample was excised from three fruits, cut into small segments (approximately 1×5 mm^2), and 9 g homogenized in 30 ml of a solution containing 100 mM Hepes/KOH (pH 7.0), 2 mM dithiothreitol (DTT) and 3% PVP-40. The homogenate was centrifuged for 20 min at $13\,000 \times g$ and aliquots of the supernatant were desalted through Sephadex G-25 columns pre-equilibrated with 10 mM Hepes/KOH (pH 7.0) and 2 mM DTT. Glycerol was added to the eluant at a final concentration of 50% and the solution stored in the refrigerator until all samples were extracted. The resulting pellets were thoroughly washed with cold 10 mM Hepes/KOH (pH 7.0). Afterwards, the pellets were incubated in 2 ml of a similar buffer solution containing 1 M NaCl for 30 min. The NaCl wash, after centrifugation, was combined with similar volume of glycerol and stored in the freezer until determination of enzyme activity.

Invertase activity was measured by incubating protein samples in a reaction mixture containing 200 mM Na-acetate buffer (pH 4.5) and 100 mM sucrose. The reactions were stopped by boiling 250 ml aliquots after the pH was neutralized to pH 7.0. Glucose (a reaction product) was determined by the glucose oxidase method of Kilburn and Taylor (1969).

Preparative isoelectric focusing was carried out in a Rotofor Preparative IEF Cell (BioRad Laboratories, Hercules, CA) according to the manufacturers specifications with a pH range between 3 and 10. Each 5 ml sample was added to 35 ml electrophoretic buffer (total of 40 ml) and subsequently electrophoresed into 20 fractions of approximately 2 ml each. The resulting pH of each fraction was measured with Hannah HI 9219 pH meter. Protein was determined following the procedure of Bradford (1976).

The potential binding of acid invertase to the cell wall was also investigated. After several water washes (4°C), the cell-wall material was incubated for 30 min in a buffered solution containing 10 mM Hepes/KOH (pH 8.0), 2 mM DTT and 1 M NaCl. The resulting slur was centrifuged and the supernatant desalted through a Sephadex G-25 pre-equilibrated with 10 mM HEPES (pH 7.0) and 2 mM DTT. The desalted solution was assayed for invertase activity immediately after.

3. Results

Desalted soluble extracts from grapefruit peel sampled at the time of harvest and after each of the three storage conditions were tested for acid invertase activity. None of the protein samples (control plus three treatments) contained detectable levels of invertase activity. However, bubbling N_2 through the protein samples from treatment IV for 2 h at 4°C (procedure recommended for the inactivation of plant invertase-inhibitors; Ewing and McAdoo, 1971) resulted in detectable levels of acid invertase activity (38 nmol/min/mg prot). In contrast, samples from control fruit similarly treated showed no ability to hydrolyze sucrose. These results alluded to the probable existence of soluble acid invertase in the flavedo cells and prompted us to further investigate its validity. The results also argued for the presence of an invertase inhibitor.

To corroborate the presence of acid invertase activity in the soluble fractions, protein samples from all treatments and control were subjected to preparative isoelectric focusing in a Rotofor Preparative IEF Cell with a pH range between 3 and 10. After electrophoretic separation, only samples from both cold treatments (III and IV) showed invertase activity. The activity eluted in aliquots corresponding to a p*I* of approximately 5.0 for both treatments III and IV (Fig. 1(a) and (b)). It is worthy of note that treatment IV (samples from chilled fruit followed by an additional 24 h at 25°C) contained considerably higher levels of invertase activity than those extracted immediately after cold treatment.

In separate experiments, the potential binding of acid invertase to the cell wall of homogenized plant material was also investigated. Direct assay of the cell-wall material from all treatments resulted in no detectable invertase activity. However, washing the cell-wall material in a buffered solution (10 mM Hepes/KOH, pH 8.0 and 2 mM DTT) containing 1M NaCl for 30 min resulted in the release of significant levels of invertase activity in treatments III and IV (Fig. 2). Lower levels of invertase activity were also detected in treatment II. Although the specific activity of the wall wash was higher than the electrophoresed samples for treatments III and IV due to lower levels of total protein in this fraction, the total amount of invertase activity recovered from the soluble fraction was an order of magnitude higher than that released from the pellets (300–700, Fig. 1, and 25 nmole sucrose hydrolyzed /min/g fw, Fig. 2, respectively).

4. Discussion

Postharvest cold treatment to Florida grapefruit is recommended for both fruit fly disinfestation and for long distance transport to foreign markets (Hatton and Cubbedge, 1982; Ismail et al., 1986). In both instances, conditioning of the fruit is necessary; otherwise the fruit may develop undesirable CI-related external

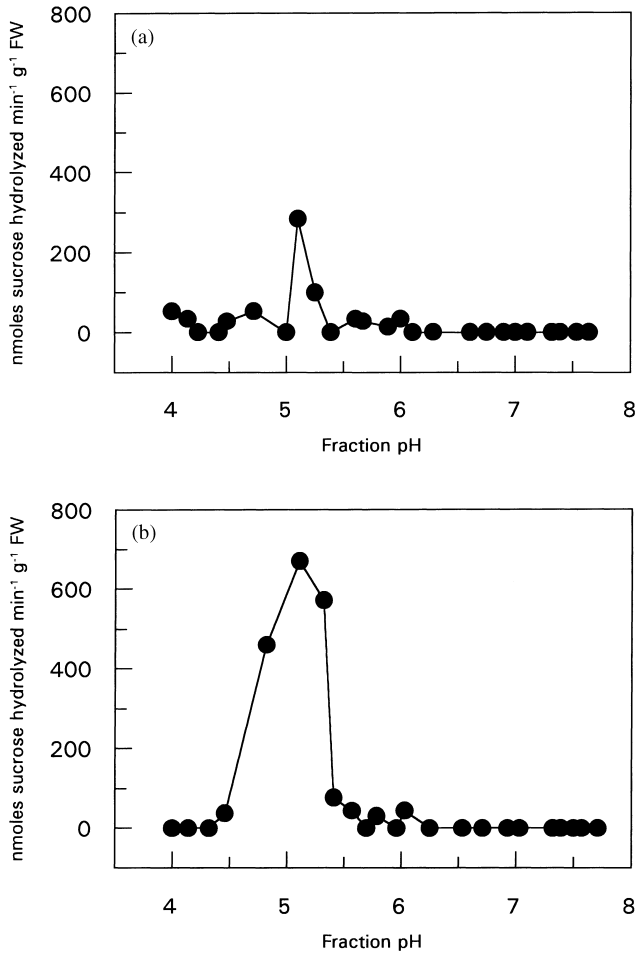

Fig. 1. Acid invertase activity profile of electrophoresed soluble extracts from grapefruit flavedo after cold treatments. Treatment III (a) and treatment IV (b). Note differences in the scale of the activities.

damage which would render the fruit undesirable for fresh marketing. Acclimation of mid-season grapefruit occurs in the field under ambient conditions in Florida. These fruit can be stored as low as 4°C immediately after harvest without suffering from CI related symptoms. Part of the CI resistance has been ascribed to the natural development of several factors, one of which involves the development of acid invertase in the flavedo cells. In late season fruit, however, such element of acclimation and CI resistance (invertase activity in the flavedo cells) is no longer present (Purvis and Rice, 1983). In the present study, we demonstrated that flavedo cells of grapefruit peel retain the capacity to re-develop acid invertase activity upon chilling long after natural deacclimation. It is

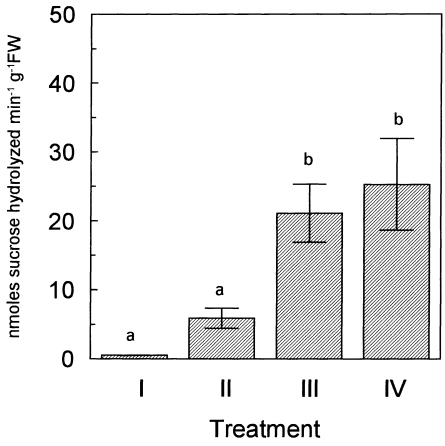

Fig. 2. Invertase activity extracted from cell-wall fractions of grapefruit flavedo washed with 1 M NaCl in a buffered solution. The NaCl containing buffered extract was desalted prior to enzyme assays. Values are the average six separate replicates ±SD. Solid bar represents the control untreated sample. $p < 0.05$.

likely that the cold-induced CI-resistance observed by Nordby and McDonald (1990) in late harvested fruit also included invertase activity as part of the overall CI-resistant process.

Invertase appears to develop during cold temperature (treatment III); however, a warming period allowed for further development of activity (treatment IV). Development of low levels of acid invertase observed in treatment II could be the result of a perceived chilling effect resulting from a rapid drop in temperature from the August field temperatures in central Florida (ca. 42°C) to 21°C storage room.

Two lines of evidence indicate the nature of invertase activity being an intracellular soluble enzyme and not a wall isoform. First, the acidic p*I* of approximately 5.0 is characteristic of the vacuolar acid invertases (Weil et al., 1994) and not of the cell-wall bound isoform (with an alkaline p*I*); and second, wall bound invertase (the second plant acid invertase) is found almost exclusively in rapidly growing tissues such as root tips or extending internodes (Strum and Chrispeels, 1990), is related to phloem unloading and sucrose partitioning (Eschrich, 1989; Miller and Chourey, 1992) and wound response (Strum and Chrispeels, 1990), and not characteristic of differentiated storage cells.

In addition, the present data suggests the probable existence of an invertase inhibitor in the flavedo cells. The considerable levels of invertase activity released after N_2 bubbling and high salt wash of the cell wall points toward this possibility. However, more detailed studies are required to acertain whether such an inhibitor indeed exists. Plant acid invertase inhibitors have been recently reported to be localized in the cell wall (Isla et al., 1992; Weil et al., 1994) although large

amounts are usually solubilized during homogenization. A wall located invertase inhibitor would be consistent with the release of the some invertase activity after high ionic wash of the wall material but, by itself, could not account for the majority of the inhibition to the soluble fraction.

In conclusion, late season grapefruit, harvested after the natural deacclimation to CI has occurred, retain the capacity to develop soluble acid invertase within the albedo cells in response to cold treatment.

References

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Eschrich, W., 1989. Phloem unloading of photoassimilates. In: Baker, D.A., Milburn, J.A. (Eds.), Transport of Photoassimilates. Longman, Harlow, UK, pp. 206–263.

Ewing, E.E., McAdoo, M.H., 1971. An examination of methods used to assay potato tuber invertase and its naturally occurring inhibitor. Plant Physiol. 48, 366.

Fuchigami, L.H., Weiser, C.J., Richardson, D.G., 1973. The influence of sugars on growth and cold acclimation of excised stems of red-oiser dogwood. J. Am. Soc. Hortic. Sci. 98, 444–447.

Hatton, T.T., Cubbedge, R.H., 1982. Conditioning Florida grapefruit to reduce chilling injury during low-temperature storage. J. Am. Soc. Hortic. Sci. 107, 57–60.

Isla, M.I., Leal, D.P., Vattuone, M.A., Sampietro, A.R., 1992. Cellular localisation of the invertase proteinaceous inhibitor and lectin from potato tubers. Phytochemistry 31, 1115–1118.

Ismail, M., Hatton, T.T., Dezman, D.J., Miller, W.R., 1986. In transit cold treatment of Florida grapefruit shipped to Japan in refrigerated van containers: problems and recommendations. Proc. Fla. State Hortic. Soc. 99, 117–121.

Kilburn, D.M., Taylor, P.M., 1969. Effect of sulfhydryl reagents on glucose determination by the glucose oxyidase method. Anal. Biochem. 27, 555–558.

Miller, M.E., Chourey, P.S., 1992. The maize invertase-deficient *miniature*-1 seed mutation is associated with aberrant pedicel and endosperm development. Plant Cell 4, 297–305.

Nordby, H.E., McDonald, R.E., 1990. Squaline in grapefruit as a possible natural protectant against chilling injury. Lipids 25, 807–810.

Purvis, A.C., Yelenosky, G., 1982. Sugar and proline accumulation in grapefruit flavedo and leaves during cold hardening of young trees. J. Am. Soc. Hortic. Sci. 107, 222–226.

Purvis, A.C., Grierson, W., 1982. Accumulation of reducing sugar and resistance of grapefruit peel to chilling injury as related to winter temperatures. J. Am. Soc. Hortic. Sci. 107, 139–142.

Purvis, A.C., Rice, J.D., 1983. Low temperature induction of invertase activity in grapefruit flavedo tissue. Phytochemistry 22, 831–834.

Purvis, A.C., Kawada, K., Grierson, W., 1979. Relationship between midseason resistance to chilling injury and reducing sugar level in grapefruit peel. HortScience 14, 227–229.

Sakai, A., Yoshida, S., 1968. The role of sugar and related compounds in variations of freezing resistance. Cryobiology 5, 160–174.

Steponkus, P.L., 1971. Cold acclimation of *Hedera helix*. Evidence for a two-phase process. Plant Physiol. 47, 175–180.

Strum, A., Chrispeels, M.J., 1990. cDNA cloning of carrot extracellular β-fructosidase and its expression in response to wounding and bacterial infection. Plant Cell 2, 1107–1119.

Wardowski, W., 1989. Cold treatment: a successful quarantine method for Florida grapefruit shipped to Japan. Packinghouse Newsletter No. 158, Florida Cooperative Extension Service, University of Florida, IFAS.

Wardowski, W., Brown, E., 1993. Postharvest decay control recommendations for Florida citrus fruit. Florida Cooperative Extension Service, Circular 359-A.

Weil, M., Krausgrill, S., Schuster, A., Rausch, T., 1994. A 17 kDa *Nicotiana tabacum* cell-wall peptide acts as an in vitro inhibitor of the cell-wall isoform of acid invertase. Planta 193, 438–445.